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McGown, A. orcid.org/0000-0002-3213-1895 and Stopford, M.J. orcid.org/0000-0002-1298-4102 (2018) High-throughput drug screens for amyotrophic lateral sclerosis drug discovery. Expert Opinion on Drug Discovery. ISSN 1746-0441

https://doi.org/10.1080/17460441.2018.1533953

This is an Accepted Manuscript of an article published by Taylor & Francis in Expert Opinion on Drug Discovery on 13/10/18, available online:
http://www.tandfonline.com/10.1080/17460441.2018.1533953

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Title: High-throughput drug screens for amyotrophic lateral sclerosis drug discovery

Abstract:

Amyotrophic lateral sclerosis (ALS) is a rapid adult onset neurodegenerative disorder characterised by the progressive loss of upper and lower motor neurons. Current treatment options are limited for ALS, with very modest effects on survival. There is a need for novel therapeutics to treat ALS and in this review we discuss the high-throughput drug screening efforts to identify novel compounds.

Areas covered:

This review highlights the many diverse high-throughput screening platforms that have been implemented in ALS drug discovery. The authors discuss cell free assays including in silico and protein interaction models. The review covers classical in vitro cell studies and new cell technologies, such as patient derived cell lines. Finally the review looks at novel in vivo models and their use in high-throughput ALS drug discovery

Expert opinions:

In the expert opinions section, the authors summarize the successes of high-throughput screening in ALS as well as the areas where future work can be directed. We also highlight new and emerging technologies, such as artificial intelligence and biomarker identification, as being critical in finding new treatments for this devastating disease.

Key Words: Amyotrophic lateral sclerosis (ALS), drug discovery, high-throughput drug screening (HTDS).

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Article highlights

- **The current treatments for patients with ALS are modest in effect and there is a need for better treatments for this devastating disease**
- **A large number of high-throughput drug screens have been performed to identify novel ALS therapeutics**
- **Screens have been performed using a wide range of different model systems, various genetic subtypes of ALS, as well as aiming for a wide range of different molecular targets and/or phenotypic outcomes - highlighting the complexity of ALS and necessity for careful study design**

Greater use of patient-derived in vitro cell models and development of better animal models of ALS will improve translation of lead compounds into clinic

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disorder that is characterised by the loss of upper and lower motor neurons (MN) in the brain and spinal cord. Patients develop progressive muscle weakness and paralysis, with death typically 2-5 years from disease onset. 90% of ALS cases are sporadic in nature, while hundreds of variants in over 30 genes are known to cause familial ALS. The most common genetic cause of ALS is a GGGGCC hexanucleotide repeat expansion in the first intron of *C9ORF72*, accounting for 40% of familial and 7% of sporadic ALS cases [1-3]. Superoxide dismutase 1 (*SOD1*) encodes an enzyme that acts as a free radical scavenger in the cell, and in 1993, was the first gene in which mutations were identified to cause ALS. *SOD1* mutations account for approximately 20-25% of familial cases [4, 5], and is the most investigated gene in the ALS field, mainly due to its early discover. Therefore, the majority of high throughput screens performed in the field have focused on mutant *SOD1*. TAR DNA-binding protein 43 (*TARDBP*) encodes TDP-43, a ubiquitously expressed RNA/DNA binding protein which is heavily implicated in ALS pathogenesis. TDP-43-positive cytoplasmic inclusions appear in the majority of both sporadic and familial ALS cases, and *TARDBP* mutations also account for 1-3% of familial ALS cases [6, 7]. Therefore, reducing TDP-43 proteinopathy has been a focus of high-throughput drug screens (HTDS).

Treatment options for ALS are limited, with the majority of treatment options palliative in nature. Currently 2 drugs are licenced for the treatment of ALS: riluzole and edaravone. Riluzole was the first FDA approved treatment for ALS and acts by blocking TTX sensitive sodium channels, inhibiting Kainate, NMDA and $GABA_A$ receptors, as well as reducing glutamate excitotoxicity [8-11]. Unfortunately riluzole is only mildly effective, with a Cochrane library review showing a 9% improvement in the probability of surviving one year [12]. Edaravone, is a free radical scavenger that was first developed as a therapeutic for ischemic stroke [13]. And Edaravone was also beneficial in the wobbler mouse model

of ALS[14] and was approved for the treatment of ALS patients in 2017 and is used in Japan, China, USA and UK. Edaravone could work by reducing oxidative stress in ALS patients, but the exact mechanisms remain unknown[15]. Unfortunately, edaravone only has mild effects in clinical trials, delaying disease progression in a subset of patients. This highlights the need for continued drug discovery efforts in ALS research to find next generation therapeutics to deliver benefits to patients.

High-throughput drug discovery is a term widely used in the scientific field to describe assays that have been optimised to handle large numbers of samples and compounds. There are no set criteria for what constitutes being a HTDS, and is model and readout dependant. Computation biology has the capacity to screen millions of compounds, cell assays in the hundreds of thousands and *in vivo* screens in the thousands. HTDS are becoming more affordable to academia with the ability to generate drug discovery pipelines, utilising robotics, liquid handling, high content imaging and data analysis pipelines. Pharmaceutical companies are also now generating strong collaborations with academia to share compound libraries for neurodegenerative research. The aim of this review is to bring together the HTDS efforts from the field and highlight the successes and strengths of the work completed. Our aim is to provide an overview of the work that has been undertaken and highlight the gaps in the field, with suggestions on how these may be addressed, to improve future study design and our understanding of this neurodegenerative disorder.

2. High-throughput Drug Screening (HTDS) in ALS

2.1. *In silico*

In silico screens and artificial intelligence (AI) technologies are an innovative and exciting way of screening drugs in an ultra-high throughput capacity. Using computational biology, millions of drugs can be tested for a predicted effect, such as binding a particular receptor or protein stabilisation, with the capacity to screen with many different virtual parameters, such as central nervous system

(CNS) penetrance and ADME properties. This throughput of screening would be near impossible in a classical lab bench setting, without a large dedicated team, making *in silico* screens a very attractive screening platform. Also the availability of commercial and open source databases that are being constantly updated, further promotes *in silico* screening as an attractive starting point for HTDS. The potential limitation of these screens is that computational systems are only as smart as the database provided to the program and the information about the compounds being tested. *In silico* libraries are improving all the time and are fast becoming the gold standard pre-screening tool for selecting more focused compound libraries for taking into classical lab bench based screens. This is of particular importance in ALS where a large failure rate of potential therapeutics in clinical trials suggests better pre-screening assays are needed.

One such *in silico* screen in ALS screened 1.5 million molecules for binding capacity at the SOD1 dimer interface [16]. The native state of SOD1 is as a dimer, and it was predicted that stabilising SOD1 as a dimer may be neuroprotective in ALS. The group identified a predicted binding site for compounds at the dimer interface and ranked the hits on the ability to bind. The 100 compounds that ranked top for binding were then validated *in vitro* for the ability to inhibit aggregation in the SOD1 carrying A4V, G85R or G93A mutations. Of these top 100 compounds, 15 showed the ability to stabilise the SOD1 dimer and prevent mutant SOD1 aggregation. The issue with finding compounds that bind strongly to proteins, is the potential for strongly binding off target proteins and/or altering the normal protein function, leading to side effects. In biology, cellular mechanisms are constantly being modulated and maintained in a fine balance, which can lead to cellular disorder and disease when the balance is shifted. To combat this, the group refined their search criteria to test a further 2.2 million compounds for the ability to dock specific SOD1 sites with limited off target binding [17]. Using a library with improved ADME properties, 6 compounds were identified that served as strong inhibitors of mutant SOD1 misfolding, with high specificity for binding SOD1 over other plasma proteins. This has highlighted new compounds for further secondary testing and optimisation, as well as highlighting the applicability of these screens in ALS research. With advances in science and

computing power, the key for these screens is for new data to be constantly added all the time and for the algorithms to evolve making these screens more powerful and informative to drive forward ALS drug discovery.

SOD1 functions normally as a dimer but monomeric SOD1 have been identified in human patients and mouse tissues. It is hypothesised that monomeric SOD1 causes toxicity - rather than the aggregates and may be an early stage of disease pathology [18, 19]. A computational screen of 4,400 drugs was undertaken for binding to two sites at the SOD1 dimer interface [20]. From this, 7 hits were taken forward for protein-protein interaction studies in A4V SOD1 mutant protein. The study identified antioxidant compounds that directly interact with SOD1 leading to stabilisation in the native state. The next stage for these drugs is further testing in cellular and animal models to show efficacy.

2.2. Cell-free assays

Misfolded proteins are a classical hallmark of ALS and protein aggregates are seen as a key indicator of disease pathogenesis. Aggregated, misfolded and mis-localised proteins are all seen in ALS and are an excellent indicator of cellular toxicity. By preventing the misfolding of proteins it is predicted that disease progression and severity could be delayed and even stopped. Evidence from Alzheimer's and other diseases argues that protein aggregates are potentially the cells way of dealing with mutant protein and are a way of removing mutant proteins, so the cell can continue to function [21, 22]. However, whilst this is still very much a hot topic, it is beyond the scope of this review.

Some HTDS in ALS have focused on modulation of protein interactions to prevent misfolding in an *in vitro* setting. These studies have the advantage of being able to investigate a single protein or protein-protein interaction and identify the mechanisms of protein misfolding due to a disease mutation, while identifying compounds that can directly modulate the protein. The ability to scale up these screens also means there is the capacity to test large libraries of compounds relatively

quickly, targeting disease relevant proteins and specific mutations, while keeping costs low. The downside of these studies is you are investigating the interaction outside of the cell, in the absence of all other interactions. Another potential factor is often these studies are performed in conditions that push the proteins towards misfolding, such as in the presence of a denaturing agent or stressor, which may lead to protein conformations not seen in disease.

Misfolding of SOD1 leads to accumulation of toxic mutant SOD1 oligomers in the spinal motor neurons [23]. This has been suggested as a pathogenic mechanism causing toxicity in the motor neurons and by reducing SOD1 oligomer levels, it may be possible to reduce neuronal toxicity. One study used the destabilising conditions of 1M guanidine hydrochloride to induce aggregate formation of mutant SOD1 (G37R) protein and investigated compounds that could inhibit the aggregation [24]. Levels of insoluble SOD1 oligomers were analysed by changes in solution turbidity and via non-reducing PAGE (Polyacrylamide gel electrophoresis). 640 FDA approved drugs were screened in this system and a group of Vitamin D derivatives and statins were identified that completely inhibited the formation of insoluble SOD1 oligomers. Unfortunately statins are known to have a strong association with functional decline in ALS patients and increased disease progression with reduced survival in SOD1 G93A mice [25, 26]. Vitamin D however has shown some positive effects in mice and patients [27, 28]. Although the drugs screened in this study did not directly identify a potential therapeutic, there is a potential to scale up the work to screen more novel compounds and find modulators of SOD1 oligomerisation.

Another therapeutic target in SOD1-ALS is the interaction between mutant SOD1 protein and Derlin-1. A previous study showed that disrupting this interaction protected MN from mutant SOD1-induced toxicity in mouse primary spinal cord culture [29]. Therefore, a cell-free HTDS assay using time resolved fluorescence resonance energy transfer (TR-FRET) technology was developed to identify compounds that disrupt mutant SOD1-Derlin-1 interaction [30]. HEK293 cells were transfected with FLAG-tagged SOD1-G93A and HA-tagged Derlin-1, and then lysed. 160,000 compounds were then

screened in the cell lysate, and the TR-FRET assay performed to identify compounds that disrupt the mutant SOD1-Derlin-1 (degradation in endoplasmic reticulum protein 1) interaction. 1,460 hit compounds were identified in the primary TR-FRET screen and taken forward. False positives were ruled out using secondary screens, and co-immunoprecipitation assays confirmed that 12 compounds directly disrupted the mutant SOD1-Derlin-1 interaction. One potent inhibitor with good drug-like properties was investigated further, and prevents 122 different types of mutant SOD1 from interacting with Derlin-1. Additionally, the inhibitor improved iPSC-MN survival from a SOD1-ALS patient carrying a L114FVX mutation, and also delayed onset and improved survival in the G93A-SOD1 mouse.

TDP-43 protein misfolding is a major pathological hallmark of ALS and aggregates are present in approximately 97% of all ALS cases [6, 31, 32]. TDP-43 is known to self-interact so an *in vitro* assay was designed to test 1,280 compounds for the ability to inhibit self-interaction and prevent aggregation [33]. Neuro2a cell lines were then used to confirm the compounds led to a reduction in insoluble TDP-43 protein. A gold containing thioredoxin, Auronofin, was identified that shifted the TDP-43 protein from an insoluble state to a soluble state. Further investigation of this class of compounds and screening of larger libraries will now be critical to further understanding TDP-43 misfolding toxicity mechanisms.

2.3. Cell-based assays

Cells are powerful models for investigating human disease, and as models for use in HTDS assays. Cells can be cultured in large numbers and are highly amenable to genetic manipulation, allowing generation of robust reporter cell lines for target-based HTDS. In addition, phenotypic HTDS using patient-derived and disease-relevant cell types, such as MN and glial cells in ALS, is now possible due to the recent advances in reprogramming technologies.

2.3.1. Reducing SOD1 transcription

Data strongly supports that mutant SOD1 protein has a toxic gain-of-toxic function. *SOD1* knockout causes no overt motor phenotype in mice [34], whilst mutant SOD1 over-expression causes MN loss and progressive paralysis in mice and rats [35, 36]. Importantly, high level mutant SOD1 expression causes these ALS like phenotypes in the rodent models, whilst low level mutant SOD1 expression does not. Accordingly, reducing SOD1 protein levels was identified as one of the earliest therapeutic targets for SOD1-ALS. Therefore, multiple cell-based screening strategies have been employed to identify compounds that reduce SOD1 protein levels. Three studies screened for compounds that reduce *SOD1* transcription [37-39], whilst others screened for compounds that inhibit SOD1 protein aggresome formation [40] or increase mutant SOD1 protein degradation [37].

Reducing *SOD1* transcription was the earliest strategy developed in a HTDS to reduce SOD1 protein levels. 116,680 compounds from 10 different libraries were screened in a stable PC12 cell line engineered to express an EGFP gene under the control of a 2.2 Kb fragment of the *SOD1* promoter [37]. 370 hit compounds were identified in the Pr^{SOD1}-EGFP reporter line, and 29 of those reduced *SOD1* transcription and were not cytotoxic. In a separate study, the Chembridge small molecule library, assembled from 30,000 predicted CNS-penetrant compounds, was screened in the same Pr^{SOD1}-EGFP reporter cell line [39]. From this library, 20 compounds reduced EGFP levels in the primary screen, although 17 compounds were cytotoxic at the effective doses and therefore discontinued from further investigation. Of the remaining 3 lead compounds, only 1 compound reduced SOD1 mRNA (messenger RNA) and protein levels in secondary experiments, and only slightly reduced SOD1 protein levels *in vivo* in mouse spinal cord. Another group generated a stable human astrocytoma-derived H4 cell line that expresses secreted luciferase under the control of a genomic *SOD1* promoter [38]. 9,600 compounds were screened in this gPr^{SOD1}-Luc reporter, and 115 hit compounds were identified and confirmed as non-cytotoxic. Using ELISA as a secondary assay, 2 of these hit compounds reduced SOD1 protein levels in a dose-dependent manner. However, the hit compounds from these three *SOD1* transcriptional repressor screens are likely to be general transcriptional

repressors, and without improving specificity toward *SOD1*, are not good candidate compounds for clinical trials.

2.3.2. Reducing protein levels or aggregation

Protein aggregation is a pathological hallmark of ALS. TDP-43 pathology appears in the majority of both sporadic and familial ALS cases [6, 7], whilst SOD1 and FUS (Fused in Sarcoma) pathologies are described in ALS patients with mutations in the respective genes [23, 41, 42]. Reducing these pathogenomic protein aggregates could therefore provide an effective ALS therapy. Therefore, several HTDS assays have been developed to identify compounds that reduces SOD1, TDP-43 or FUS aggregation [37, 40, 43-45]. However, it is still unknown whether the protein aggregation is toxic or protective in affected cells.

To model SOD1 protein aggregation in a HTDS, COS1 cells were transduced with adenovirus expressing a mutant G85R-SOD1-GFP fusion protein and then treated with a proteasome inhibitor [40]. In this system, the G85R-SOD1-GFP fusion protein formed SOD1 aggresomes. 20,000 compounds were screened, and 12 compounds reproducibly inhibited aggresome formation. In addition to the *SOD1* transcriptional reporter line (described in section 2.3.1.), Broom et al. also generated a stable PC12 cell line with inducible G37R mutant SOD-EGFP fusion protein expression [37]. The commercially available Prestwick and MicroSource libraries (640 and 1040 compounds respectively) were screened in this $SOD1^{G37R}$-EGFP line to identify compounds that reduce G37R-SOD1-EGFP protein levels. 67 hit compounds reduced EGFP fluorescence, but the majority were either cytotoxic or failed to replicate in dose-response assays. Authors describe further analysis was on going for 2 of the compounds.

TDP-43 and FUS are predominantly localised in the nucleus, but under cellular stress, both proteins can translocate to the cytoplasm and associate with stress granules [46, 47]. Interestingly, ALS-causative mutations in *TARDBP* and *FUS*, increase the propensity for their encoded proteins to aggregate [5, 48]. As such, aberrant stress granule dynamics could drive TDP-43 and FUS aggregation and

proteinopathy, and could represent a key initiating event in ALS pathogenesis [49]. Therefore, three HTDS assays were developed to screen for compounds that inhibited TDP-43 or FUS-positive stress granules.

The first used a stable PC12 cell line with inducible expression of a TDP-43-GFP fusion protein, [43], whilst the later 2 HTDS used human-derived cells, which are covered later in section 2.3.6. Under basal conditions, the PC12 reporter cells had diffuse nuclear TDP-43-GFP localisation. However, TDP-43-GFP localised to nuclear aggregates and cytoplasmic stress granules after arsenite-induced oxidative stress, and this phenotype was robust enough to take forward to the HTDS. A library of approximately 75,000 compounds was screened in this TDP-43-GFP positive stress granule assay, and included selected FDA-approved drugs, natural products, and commercially available compounds to maximise chemical diversity. 16 compounds were identified from the primary screen that significantly reduced TDP-43-GFP positive stress granule formation, were validated in secondary dose-response assays, and did not induce cellular toxicity or affect TDP-43 expression levels. Hit compounds were then tested in WT-TDP-43 and A315T mutant TDP-43 C.elegans lines, and one lead compound ameliorated TDP-43 induced MN loss and motor deficits. This is an interesting avenue of discovery and further work is needed to elucidate the mechanisms of stress granule formation and how inhibition of their formation may lead to neuroprotection.

2.3.3. Anti-glutamatergic

Glutamate excitotoxicity - caused by excessive glutamate in the synaptic cleft is implicated in MN injury and death in ALS[50]. One potential therapeutic approach to protect against excitotoxicity is to enhance synaptic glutamate reuptake by glial cells. The glial glutamate transporter (GLT-1: also known as EAAT2 and SLC1A2) plays a major role in glutamate clearance, and two HTDS were developed to identify compounds that upregulate GLT-1 expression. In the first a structurally diverse library of 1,040 FDA-approved drugs and nutritionals were screened in organotypic spinal cord slice cultures prepared from postnatal day 9 rats [51]. After 5-7 days treatment, tissue was harvested for

GLT1 protein levels, and the top hit compounds were enriched for beta-lactam antibiotics. Foetal astrocytes or COS7 cells transfected with a 2.7 kb *GLT-1* promoter fragment linked to a reporter were used to confirm that the beta-lactam compounds increased GLT1 expression. A lead compound also increased GLT-1 protein and function *in vivo*, and slowed disease progression and extended survival in the G93A-SOD1 mouse model of ALS.

In the second GLT-1 screen, a high throughput enzyme-linked immunosorbent assay (ELISA) was used to measure GLT-1 protein levels in a stable rat primary astrocyte line with high expression of human GLT-1 [52]. Approximately 140,000 small molecules were screened, including FDA-approved drugs, purified natural products, commercially available compounds, and small molecules procured from academic institutions. 293 compounds increased GLT-1 expression in the primary screen, and 61 compounds showed a dose-response in secondary assays. 3 compounds with high potency, low toxicity, and chemical tractability were taken forward for lead optimisation. However, three anti-glutamatergic compounds - memantine, talampanel, and ceftriaxone – all failed to show clinical efficacy in advanced-stage ALS clinical trials suggesting excitotoxicity modulation alone is insufficient for protection in ALS [53-56].

2.3.4. Reducing oxidative stress

Elevated levels of oxidative damage in pathologically affected areas of the CNS in ALS patients implicates oxidative stress in ALS pathogenesis [57]. Therefore, antioxidant compounds have been highlighted as potential ALS therapeutics. The 2,000 compound Spectrum Collection of known drugs and natural products was screened for compounds with protective antioxidant properties in two separate HTDS [58, 59]. In the first HTDS, oxidative stress was induced in NSC34 cells by serum withdrawal, and DCF (Dichlorofluorscein) fluorescence was used to measure reactive oxygen species (ROS) levels [58]. 164 compounds were identified in the primary screen that significantly reduced ROS. These hits were further refined in subsequent assays and 2 compounds were identified that were neuroprotective, well tolerated *in vivo*, have good predicted CNS penetrance and enhanced drug-like

properties. In the second HTDS, the Spectrum Collection was screened for compounds that upregulate activity of NRF2 (nuclear factor erythroid 2 – related factor 2) – a transcription factor that binds the antioxidant response element (ARE) and drives expression of antioxidant enzymes [59]. CHO cells were stably transfected with an ARE-EGFP construct, thereby reporting NRF2 activity. 44 NRF2 activating compounds were identified in the primary screen, and of those, 17 were neuroprotective and non-cytotoxic in secondary assays, and were predicted to have good CNS penetrance. Encouragingly, anti-oxidant free radical scavenger edaravone has demonstrated efficacy in phase 3 clinical trials [60], and suggests anti-oxidant compounds identified in these HTDS may also translate to clinic.

2.3.5. Cytoprotection

A key pathophysiological event in ALS is MN degeneration and loss. Therefore, several studies have screened to identify compounds that improve MN survival. In the first HTDS, MN from embryonic rat spinal cords were purified, cultured under trophic deprivation and then stained using calcein-AM to identify live cells [61]. Approximately 40,000 compounds were screened in this phenotypic assay, and olesoxime was one of the most potent neuroprotective compounds. Additionally, olesoxime increased MN survival in a dose-dependent manner and also improved neurite outgrowth and axonal branching. Further, olesoxime was neuroprotective and neuroregenerative in two rodent models of nerve injury, and delayed onset and improved survival in G93A-SOD1 mice. However, olesoxime did not provide efficacy in advanced stage clinical trials of ALS [62].

As described in section 2.3.1., mutant SOD1 likely confers toxicity through a gain-of-function mechanism, and therefore one HTDS was developed to identify compounds that ameliorate this toxicity. PC12 cells stably expressing mutant (G93A or G85R) SOD1 protein tagged with a yellow fluorescent protein (YFP) were used[63]. In the primary screening assay, PC12 G93A-SOD1-YFP cells were treated with the proteasome inhibitor MG132, which caused mutant SOD1 protein-induced cell death. Over 50,000 compounds were screened in this SOD1-toxicity assay, and compounds were

selected to maximise chemical structural diversity and to include biologically well-characterised compounds (including FDA-approved and clinically tested compounds). 68 compounds improved cell viability, and only one false positive was identified. Secondary screening in the PC12 G85R-SOD1-YFP model confirmed that the vast majority of these hit compounds also inhibited SOD1-YFP aggregation. Chemoinformatic methodologies identified several chemical scaffolds or chemotypes for further testing in the aforementioned HTDS assays. As a result, 3 chemical scaffolds with good drug-like properties were proposed for lead optimisation to identify candidate compounds to test in the G93A-SOD1 mouse model, and then translate into clinical trials.

WT murine embryonic stem cells (mESC) or mESC carrying a *SOD1*^{G93A} transgene were used in another SOD1-toxicity HTDS [64]. Both mESC lines also carry *GFP* under control of the MN-specific promoter Hb9, and were differentiated to produce Hb9-GFP+ MN for the HTDS assay. Hb9-GFP+ MNs were also cultured under neurotrophic factor withdrawal to induce MN death. Approximately 5,000 compounds (including known drugs, bioactives, and chemically annotated compounds) were screened in both WT and G93A-SOD1 Hb9-GFP+ MNs to identify compounds that improve MN survival. Hit compounds from the primary screen were re-tested in a secondary dose-response assay. The GSK-3 inhibitor kenpaullone was the most protective compound tested in both WT and mutant MN, and was taken forward for further analysis. The compound also reduced mutant SOD1 protein levels in G93A-SOD1 MN, and improved MN survival in cultures derived from iPSC from *TARDBP*-ALS, *SOD1*-ALS as well as from a heathy control. Critically, this HTDS identified the HGK-Tak1-MKK4-JNK-c-Jun cell death signalling cascade as a potential therapeutic target in ALS.

2.3.6. Patient/Human-derived cell models

For a long time, scarcity of human cells from the CNS has posed a serious challenge to studying and understanding complex neurodegenerative disorders such as ALS. However, over the last 12 years, ground-breaking human cell reprogramming technologies have advanced rapidly. Human somatic cells can now be reprogrammed to induced pluripotent stem cells (iPSC) [65, 66], which can

then be differentiated into neuronal progenitor cells (NPC), and subsequently into neuronal and glial cells [67]. More recently, transdifferentiation methologies allow the generation of NPCs directly from fibroblasts [68-70]. As such, reprogramming technologies present drug discovery scientists with a readily available source of ALS patient derived neurons and glial cells that retain key pathophysiological properties of ALS, and can be utilised for pathophysiologically relevant phenotypic drug screening.

Burkhardt et al., were the first group to use ALS patient derived cells in a phenotypic HTDS assay [45]. In the study, fibroblasts from 10 healthy controls, 8 familial ALS patients, and 16 sporadic ALS patients were reprogrammed into iPSC. All iPSC lines were then differentiated into MN (iPSC-MN), and characterised for TDP-43 proteinopathy. In iPSC-MN from 3 of the sporadic ALS patient lines, neurons contained spontaneous intranuclear TDP-43 aggregates that were hyper-phosphorylated and morphologically similar to TDP-43 aggregates seen in ALS patient post-mortem CNS [6, 31, 71]. Although intranuclear TDP-43 aggregates are less commonly described in ALS post-mortem tissue, one of the sporadic ALS patients displays the intranuclear TDP-43 aggregates both *in vitro* in the iPSC-MN and post-mortem in spinal cord and frontal lobe neurons. Using one of the sporadic ALS patient iPSC-MNs that display nuclear TDP-43 inclusions, a HTDS was developed to identify compounds that could reduce TDP-43 aggregation. 1,757 compounds were screened, and 38 hit compounds reduced the percentage of iPSC-MN cells that contained TDP-43 aggregates. The hits were tested on cortical neurons derived from the same ALS patient iPSC in a secondary dose-response assay, and 4 classes of compounds were identified that reduced TDP-43 aggregates in a dose-dependent manner.

In another HTDS, CRISPR-Cas9n (clustered regularly interspaced short palindromic repeats) gene editing technology was used to generate iPSC reporter cell lines with an ALS causative *FUS* mutation [44]. The iPSC were derived from a healthy donor control, and eGFP was inserted into the C-terminal domain of genomic *FUS*. Additionally, isogenic iPSC lines were generated with or without the ALS-

causative P525L mutation in genomic *FUS*. The P525L mutation lies in the nuclear localisation signal (NLS) of FUS, and increased cytoplasmic FUS mislocalisation in the iPSC. The P525L mutation also increased FUS-eGFP recruitment to stress granules after sodium arsenite treatment, and formed the basis of the HTDS assay. Approximately 1,000 compounds against known targets were screened in the P525L-FUS-eGFP iPSC to identify compounds and molecular pathways that affect stress granule dynamics. 96 hit compounds reduced FUS-eGFP+ stress granule area in the primary screen, and 13 hits inhibited the PI3K/AKT/mTOR (mammalian target of rapamycin) signalling pathway – which inhibits autophagy and was assessed in secondary experiments. In neuronal cultures derived from the P525L-FUS-eGFP iPSC lines, autophagy stimulation using mTOR inhibitors ameliorated stress granule dysfunction, reduced neuronal apoptosis, and improved axonal branching and health. Further, genetic upregulation of autophagy extended lifespan and ameliorated retinal neurodegeneration in transgenic drosophila models expressing WT or mutant FUS in the CNS or retina respectively. This study corroborates a link between autophagy and stress granule dynamics, and highlights autophagy stimulation as a potential therapeutic target in ALS. Finally, a second HTDS was performed in the P525L-FUS-eGFP iPSC stress granule assay using approximately 1,600 US Drug Collection MicroSource Discovery compounds to identify clinically approved drugs that could reduce the FUS-ALS stress granule phenotype. Incredibly, 70 hit compounds were identified, that included a number of brain-penetrant anti-psychotics and anti-depressants that are known autophagy inducers. Importantly, 2 of these clinically approved compounds are brain-penetrant and share a chemical scaffold that could be taken forward for lead optimisation.

The third HTDS using patient-derived cells assayed for iPSC-MN survival [69]. iPSC-MN derived from ALS patients had worse survival than iPSC-MN derived from healthy donor controls, and this pathophysiologically-relevant phenotype was developed into a HTDS assay. 1,416 compounds, including clinically approved drugs and compounds undergoing clinical testing, were screened in iPSC-MN from a SOD1-ALS patient carrying a L114FVX mutation. 27 hit compounds improved MN

survival in the primary screen, and 14 of these compounds target the Src/c-Abl signalling pathway. Genetic knockdown of Src and c-Abl using siRNA also improved SOD1-ALS iPSC-MN survival, warranting further investigation into this pathway. Several Src/c-Abl inhibitors were also validated in dose-response MN survival assays, and bosutinib – which had the lowest EC50 - was taken forward for further analysis. Bosutinib also improved iPSC-MN survival in cells derived from 8 familial ALS patients (containing mutations in *SOD1*, *C9ORF72*, or *TARDBP*) and also in 2 of 3 sporadic ALS patients. Finally, bosutinib also delayed onset, improved survival, increased spinal MN counts, and reduced misfolded SOD1 in the G93A-SOD1 mouse.

The final HTDS using patient-derived cells also assayed for improved iPSC-MN survival, in addition to other relevant phenotypes[73]. iPSC-MN derived from ALS patients displayed 'ALS phenotypes' including reduced neurite length, increased caspase 3 cleavage, increased LDH leakage, increased number of stress granules, and had FUS or TDP-43 aggregates – dependent on genetic subtype – compared to iPSC-MN derived from healthy controls. 1,232 approved drugs were initially screened in *FUS*-ALS iPSC-MN cultures in which four of these ALS phenotypes were measured. 95 compounds rescued the four pathophysiologically relevant phenotypes measured, and the compounds were re-tested in a similar multi-phenotypic dose-response assay using *TARDBP*-ALS iPSC-MN. Consequently, nine compounds were identified that improved the ALS phenotypes, and ropinirole – a dopamine D2 receptor agonist - was selected as the lead candidate based on brain-penetrance, side effects, and dose-response. Additionally, ropinirole corrected these ALS phenotypes in 16 out of 22 iPSC-MN derived from different sporadic ALS patients. Identification of genomic or transcriptomic signatures – or other clinical phenotypes or biomarkers - that could differentiate the ropinirole responders from non-responders could allow patient stratification in potential ropinirole clinical trials – setting a precedent in ALS clinical trials and drug discovery.

2.4 *In vivo*

In vivo screens are one of the most challenging models to develop and optimise for HTDS. Comparing different animal models of ALS is beyond the scope of this review, but is expertly covered in [74]. *In vivo* models allow the generation of multiple data sets such as behaviour, survival and the collection of different tissue types meaning large cohorts of data can be generated from each study. The negative side is that model generation and validation HTDS can be difficult and time consuming, making *in vivo* HTDS increasingly difficult. Often large numbers of animals are required for each compound as well as large quantities of drugs and lengthy exposures, making study design and cost effectiveness difficult. To combat this, large screening studies have moved away from the classical murine models to use novel animal models such as drosophila, c.elegans and zebrafish to perform HTDS. These models maintain the cellular complexity of larger mammalian models but have the advantages of being cheaper, developing faster and being more amenable to developing large scale screening studies. The downside is that these models are often evolutionarily and genetically further away from mammalian models, but act as excellent *in vivo* systems for pre-screening large numbers of compounds, before testing in mammalian models.

C.elegans are nematode worms with a simple nervous system, consisting of 302 neurons and 50 glial cells, alongside a fully sequenced genome. Their small size, conserved genetics and rapid development make them an excellent model for developing HTDS assays. C.elegans carrying a *TARDBP* mutant were used to screen 3,850 compounds in a phenotypic screen for improving motor behaviour [75]. C.elegans were pooled and treated with compounds for 6 hours before the group looked for a rescue effect on motor behaviour. From the 13 positive hits identified, the group then screened in zebrafish models expressing mutant TDP-43, SOD1 or FUS to confirm that compounds improved motor behaviour. Ten hits were identified in the zebrafish screen, which included a family of neuroleptic compounds. The most potent hit, pimozide, a T-type Ca2+ blocker, was then tested in the SOD1 G93A mouse and in a small scale human clinical trial where treatment with pimozide confirmed stabilisation of motility in patients. This work is an excellent example of how screening in

invertebrates and lower organisms can be a powerful pre-screening tool for therapeutic effect before confirming the positive effect of compounds in mammalian models.

Another commonly used laboratory *in vivo* model is the drosophila. Drosophila are a small fruit fly with a well-defined developmental pathway, rapid development, conserved genetics and fully sequenced genome, allowing the generation of specific mutant models of neurological disorders. Mutant TDP-43 causes pupal larval lethality in drosophila, and a therefore, 1,200 compounds were screened in a mutant TDP-43 drosophila model to identify compounds that could ameliorate this phenotype [76]. The PPAR (peroxisome proliferator activated receptors) agonist, pioglitazone, was identified as neuroprotective, rescuing locomotor defects and extending larval survival in the drosophila. It was also shown that pioglitazone does not extend survival in adult mTDP-43 or mFUS drosophila, but has neuroprotective effects, highlighting PPAR's as a potential therapeutic target for further investigation. Pioglitazone was shown to protect against toxicity in a tissue specific manner, with protection of glial cells, but not muscle. Interestingly the drug had no effect on SOD1 mutants highlighting the ability for these models to identify therapeutic agents with mutation specific effects. This highlights the power of *in vivo* studies to give large amounts of information, identifying that a drug is having a neuroprotective effect but that it was cell specific.

Zebrafish are another widely used *in vivo* model due to their rapid development, conserved genetics and applicability to HTDS. A high-throughput screening method has been developed using zebrafish embryos to screen for potential ALS therapeutics. A mutant sod1 G93R zebrafish model was utilised to screen 2,000 compounds for their ability to reduce mutant SOD1 toxicity [77]. The sod1 zebrafish model contains an hsp70-DsRed construct which allows the visualisation of cellular stress as an increase in DsRed fluorescence. Drug effect was measured by looking for compounds that showed reduced DsRed, indicative of a reduction in neuronal stress. This screen has the capacity to screen over 500 compounds per week with over 90% specificity and sensitivity. 7 compounds were found to strongly reduce neuronal stress and a lead compound which is neuroprotective in the zebrafish, is

now being tested in the SOD1 mouse mode (unpublished data). *In vivo* drug screens have the potential to be very powerful and deliver large amounts of data. Study design and assay optimisation is a critical step when designing *in vivo* screens and choosing the correct model to answer your research hypothesis is imperative.

Conclusions:

ALS is a rapidly progressive neurodegenerative disease caused by a wide range of complex genetic and environmental factors. Riluzole and edaravone are currently the only two clinically approved compounds for ALS treatment - both of which only have a modest effect on disease progression and survival. Therefore, there is a desperate need to identify new efficacious compounds, and a variety of high-throughput drug screens have been developed and undertaken in the ALS field to achieve this. These screens have used a wide range of different model systems, modelling various genetic subtypes of ALS, as well as aiming for a wide range of different molecular targets and/or phenotypic outcomes. A summary of all the trials is available in table 1. This highlights the complexity of ALS but also the huge amount of drug discovery research that has already been undertaken for ALS therapeutics.

Expert Opinions

HTDS are powerful starting points in drug discovery to identify novel compounds and chemical structures as therapeutic candidates. However, the vast majority of clinical trials investigating potential ALS therapeutics have failed to show clinical efficacy[74], and these failures include candidate compounds identified from HTDS. Therefore, we need to reassess how best to implement HTDS in the ALS drug discovery process. We believe advancements in disease understanding, computational disease modelling, animal and patient derived cell models, as well as increased application of artificial intelligence and patient stratification, will improve the identification and translation of lead

compounds from HTDS into clinical efficacy.Phenotypic, genetic, and pathophysiologic heterogeneity make ALS an incredibly complex disease.

Mutations in over 30 genes are known to cause ALS, and these different mutations are likely to drive different pathogenic pathways that culminate in MN degeneration and manifest as an ALS phenotype. The heterogeneity of the disease has likely contributed to the poor success of clinical trials, and currently there is no single well defined therapeutic target for the total ALS population. Therefore, we predict phenotypic screening - which does not require a pre-defined molecular mechanism of action (MMOA) – will be more productive than target-based screening in identifying efficacious lead compounds for ALS as a whole population. As such, the advance of patient-derived *in vitro* cell models that recapitulate pathophysiological aspects of ALS will be key models for HTDS. In addition, generation and development of better animal models of ALS - particularly mouse models where therapeutic identification has had a high failure rate in clinical trials - are needed. These patient-derived *in vitro* and new *in vivo* models are the future gold standard models for HTDS in ALS.

Additionally, given the heterogeneity, certain therapeutics may only work on specific genetic or pathophysiologic subtypes of ALS, and multiple different precision medicine strategies may be necessary to treat the whole ALS population. To demonstrate this, post-hoc meta-analysis was performed on data from multiple ALS clinical trials of lithium carbonate [79]. Lithium carbonate was not efficacious in the total ALS population; however, lithium carbonate did improve survival in a genetic subtype of patients carrying *UNC13A* mutations. In addition, SOD1 antisense oligonucleotide therapy[80], arimoclomol[81] and pyrimethamine[82] may provide efficacy specifically in ALS patients with *SOD1* mutations. In future, better patient stratification should help assign efficacious therapeutics to the correct patients and ALS subtypes. These breakthroughs are most likely to come from improved genetic screening and biomarker development, which will allow for more specific personalised medicine approaches. This will hopefully lead to improved therapeutic effects and improved success rates in stratified clinical trials.

Also, ALS therapeutics will only provide efficacy if they reach the CNS by crossing the blood-CNS barrier (B-CNS-B). Multiple studies implicate B-CNS-B impairment in ALS patients, which likely impedes drug delivery into the CNS [83]. For example, perivascular collagen IV accumulation seen in SALS patients - but not in SOD1 mouse models of ALS – may attribute to poor drug delivery to the CNS [84]. Therefore, animal models that more accurately model the B-CNS-B of ALS pathophysiology will give a more accurate prediction of CNS penetrance of potential ALS compounds.

Lastly, artificial intelligence has the potential to improve the efficiency of drug discovery at many stages of the pipeline. Scientists cannot accurately assimilate, process or recall the sheer amount of scientific data in their own disease's field, let alone the whole scientific research field, that has been generated over the last few decades. However, AI technology is being developed to digest and interpret this information, and make 'hidden knowledge' usable to researchers. As a result, AI will improve target selection for HTDS and aid lead compound optimisation. Additionally, with greater genetic characterisation of ALS patients recruited to clinical trials, AI may help identify responsive genetic subtypes of patients from clinical trials.

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Stars denote journals of:

*** Journal of interest**

****Journal of considerable interest**

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Model	number of compounds in primary screen	Lead hits	reference and year	Ref No
Computational	1,500,000	15 hits - available in orginal paper	Ray et al, 2005	16
Computational	2,200,000	6 hits - available in original paper	Nowak et al, 2010	17
Computational	4,400	quercertin, quercetin 3-beta-d-glucoside, and epigallocatechin gallate (EGCG)	Ip et al, 2017	20
Protein interaction	640	Vitamin D derivatives and statins	Anzai et al, 2016	24
Protein interaction	160,000	Unknown	Tsuburaya et al, 2018	30
Protein interaction	1,280	auronofin, chelerythine and riluzole	Oberstadt et al, 2018	33
PC12 cell line	116,680	Unknown	Broom et al, 2006	37
Astrocytoma derived H4 cell line	9,600	2 hits - structure available	Murakami et al, 2011	38
PC12 cell line	30,000	N-{4-[4-(4-methylbenzoyl)-1-piperazinyl]phenyl}-2-thiophenecarboxamide	Wright et al, 2012	39
COS1 cell line	20,000	Cardiac glycosides, scriptaid, and DPD ((5-(3-Dimethylamino-propylamino)-3,10-dimethyl-10H-pyrimidol[4,5-b]quinolone-2,4-dione)	Corcoran et al, 2004	40
PC12 cell line	75,000	One unnamed compound	Boyd et al, 2014	43
iPSC	1,000	96 hits including 13 PI3K/AKT/mTOR pathway inhibitors	Marrone et al, 2018	44
iPSC	1,600	paroxetine, promethazine, and trimipramine	Marrone et al, 2018	44
iPSC-derived motor neurons	1,757	38 hits - including 4 different classes of compounds	Burkhardt et al, 2013	45
rat spinal cord slice cultures	1,040	beta-lactam compounds	Rothstein et al, 2005	51

rat primary astrocyte line	140,000	293 hits - 3 selected for lead optimisation	Colton et al, 2010	52
NSC34 cell line	2,000	CAPE and esculetin	Barber et al, 2009	59
CHO cell line	2,000	andrographolide and s[+]apomorphine	Mead et al, 2013	59
embryonic rat spinal cord derived motor neurons	40,000	olesoxime	Bordet et al, 2007	61
TDP-43 Phenotypic screen	50,000	arylsulfanyl pyrazolones, cyclohexane-1,3-dione, and pyrimidine 2,4,6-trione	Benmohamed et al, 2011	63
murine embryonic stem cell derived motor neurons	5,000	kenpaullone	Yang et al, 2013	64
iPSC-derived motor neurons	1,416	bosutinib	Imamura et al, 2017	83
iPSC-derived motor neurons	1,232	ropinirole	Fujimori et al, 2018	73
C.elegans	3,850	neuroleptics - pimozide	Patten et al, 2017	75
Drosophilla	1,200	piolglitazone	Joardar et al, 2015	76
Zebrafish	2,000	Unknown	mcgown et al, 2016	77